Minimal Residual Disease is a Prognostic Marker for Neuroblastoma With Bone Marrow Infiltration

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Objective: This pilot study focused on whether flow cytometry (FCM) detection of minimal residual disease in bone marrow (BM) could predict the outcome of patients with advanced neuroblastoma (NB).

Patients and Methods: Fifty-seven stage 4 NB patients with BM infiltration were enrolled in this study. All of them received NB-2001 protocol. BM samples were examined for tumor cell contamination by both morphology and FCM with CD45-FITC$^-$/CD81-PE$^+$/CD56-PECy5$^+$ monoclonal antibodies cocktail at diagnosis and after 4 courses of chemotherapy.

Results: BM samples of all patients were positive at diagnosis by FCM, and samples from 30 patients became negative after 4 courses of chemotherapy, 10 patients relapsed (33.3%) in mean 45.5 months, range 7 to 69. Another 27 patients remained positive, and 20 of them relapsed (74.1%) in mean 24.2 months, range 8 to 48. There was a statistically significant difference in event-free survival between the 2 groups ($P = 0.002$).

Conclusions: Persistence of minimal residual disease in BM may work as a chemotherapy response marker and predict the prognosis in advanced NB.

Key Words: neuroblastoma, bone marrow, minimal residual disease, flow cytometry, prognosis

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Neuroblastoma (NB), the most common extracranial pediatric solid tumor, remains a clinical enigma. Myeloablative therapy, especially if followed by 13-cis-retinoic acid, has improved event-free survival (EFS) of high-risk NB to approximately 40% at 4 years from diagnosis.[1–3] Although substantial improvement in outcome of certain well-defined subsets of patients has been observed during the past few decades, the outcome for children with a high-risk clinical phenotype has improved only modestly, with long-term survival still <30% for those >18 months of age at diagnosis with metastatic disease despite aggressive multimodality therapy.[4] Most stage 4 NB patients have bone marrow (BM) metastases at diagnosis, and BM is a frequent site of recurrent disease. As BM infiltration is frequently seen in NB patients, the detection of tumor cells at this site is a prerequisite for correct staging and monitoring of response to therapy. The search for BM infiltration is therefore of utmost importance for both staging and therapeutic purposes. Morphologic screening of BM aspirates, based on the identification of clumps or rosettes of tumor cells, has been the gold standard for long time.[5] Considering a detection level of 1% tumor cells for cytologic and/or histologic screening of BM samples,[6] it seems conceivable that morphologic techniques alone were not regarded as a sensitive enough method to monitor minimal residual disease (MRD). For this reason, some new methods have been developed through the last 2 decades. Immunocytology, reverse transcriptase-polymerase chain reaction (RT-PCR), and flow cytometry (FCM) are the most frequently applied techniques for detecting MRD in NB patients.[7–9] This study was carried out to determine whether MRD in BM detected by FCM could predict the long-term prognosis.

We have earlier reported that the FCM detection of MRD in BM have shown a considerable higher sensitivity than morphology and may highly affect the prognosis of the disease.[10] This study was aimed at assessing the long-term prognostic significance of MRD detected by FCM in stage 4 NB with BM involvement after 4 courses of chemotherapy which indicated the chemotherapy response.

MATERIALS AND METHODS

Patients

Fifty-seven patients, determined as stage 4 NB based on International Neuroblastoma Staging System at initial presentation and found with BM infiltration by FCM, were enrolled in this study between 2002 to September 2009. All patients received the therapy based on our hospital protocol NB-2001 (Fig. 1). Complete response (CR) or very good partial response (VGPR) was considered as favorable response. Table 1 lists internationally proposed criteria to determine response to therapy in patients with NB. Fifty-one of the 57 patients (89.4%) succeeded in obtaining

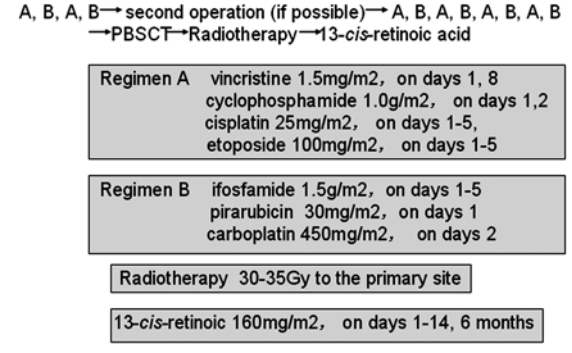
A, B, A, B→ second operation (if possible)→ A, B, A, B, A, B, A, B
→PBSCT→Radiotherapy→13-cis-retinoic acid
Regimen A vincristine 1.5mg/m2, on days 1, 8
cyclophosphamide 1.0g/m2, on days 1,2
cisplatin 25mg/m2, on days 1-5,
etoposide 100mg/m2, on days 1-5
Regimen B ifosfamide 1.5g/m2, on days 1-5
pirarubicin 30mg/m2, on days 1
carboplatin 450mg/m2, on days 2
Radiotherapy 30-35Gy to the primary site
13-cis-retinoic 160mg/m2, on days 1-14, 6 months

FIGURE 1. Neuroblastoma (NB-2001) protocol for patients with advanced neuroblastoma. PBSCT indicates peripheral blood stem cell transplantation.

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TABLE 1. Definitions of Response to Treatment

Response	Primary	Metastases
CR	No tumor	No tumor (chest, abdomen, liver, bone, bone marrow, nodes, etc.)
VGPR	Reduction >90% but <100%	No tumor (as above except bone); no new bone lesions, all preexisting lesions improved
PR	Reduction 50% to 90%	No new lesions; 50% to 90% reduction in measurable sites; bone marrow samples with tumor; bone lesions same as VGPR

CR indicates complete response; PR, partial response; VGPR, very good partial response.

CR/VGPR after chemotherapy and surgery. Six of 57 patients never reached CR or VGPR and 4 of them progressed. Twenty-five patients in total (43.9%) received peripheral blood stem cell transplantation (PBSCT) and the others did not because of the financial limitation. For the entire cohort, total 30 patients progressed or relapsed till now.

In brief, the protocol NB-2001 (Fig. 1) are consisted of 2 alternate chemotherapy cycles termed A and B, surgical resection, radiotherapy for local control, PBSCT, and 13-cis-retinoic acid. This protocol was designed with flexible courses of chemotherapy based on therapy response. There are another 4 more courses of chemotherapy after achieving CR/VGPR and negative BM MRD, but no more than 18 courses. Up to June 2010, the median follow-up for survivors was 24.2 months (range, 7 to 69 mo).

Another 8 patients with BM infiltration were ruled out from this study because they dropped off the protocol by the parent's decision to stop the therapy after 1 to 2 courses of chemotherapy.

Written informed consent was obtained from the parents or guardian of the patients and all aspects of the investigation were approved by the institutional review boards.

Flow Cytometric Analysis

NB-MRD detection by FCM with CD45-FITC/CD81-PE/CD56-PECy5 has been established in 2002 at Shanghai Children's Medical Center and the details of this method have been described earlier.[10] The sensitivity and reliability were established by human NB cell line TGW. This system enabled the accurate detection of TGW in 10^4 cells, therefore the sensitivity is 0.01%. If the cell with CD45-FITC/CD81-PE/CD56-PECy5 was more than 0.01% in BM sample, it would be considered positive. All the patients detected the NB-MRD by FCM before therapy, after 4 courses and/or before surgery. At the same time, the morphology evaluation was also done.

Statistical Analysis

The statistical analyses were carried out with SPSS 15.0 for Windows software. Data concerning follow-up were summarized using mean and ranges. EFS is taken as the time from diagnosis to the date of disease relapse. Surviving patients are censored at the time of last follow-up. The probabilities of EFS and 95% confidence intervals (CI) were estimated by the Kaplan-Meier method, and survival comparison was made between groups by the log-rank test. Prognostic factors were analyzed using a Cox proportional hazards analysis. In statistical tests, a 5% level was taken to indicate significance.

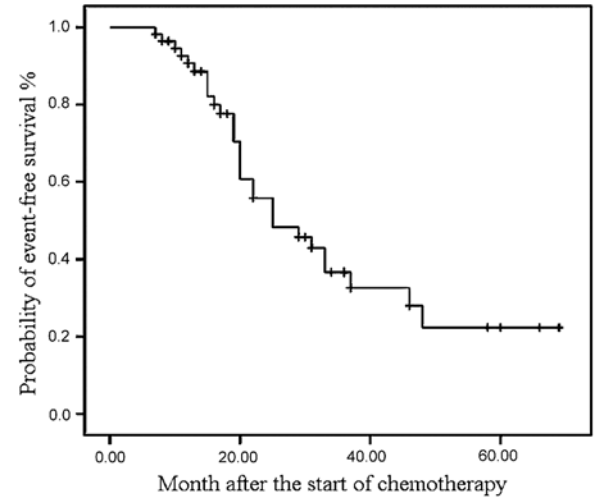

FIGURE 2. Kaplan-Meier plots for event-free survival in patients with bone marrow infiltration neuroblastoma.

RESULTS

The overall 5-year EFS of 57 NB stage 4 patients with BM metastatic disease is 22.4%, with a mean survival time 34.6 months (ranging from 7 to 69, 95% CI, 27.8-41.3) (Fig. 2).

Of the 57 patients, 30 became MRD negative in BM by FCM after 4 courses of chemotherapy with a mean follow-up of 45.5 months (ranging from 7 to 69, 95% CI, 35.3-55.8). Another 27 patients with a mean follow-up of 24.2 months (ranging from 8 to 48, 95% CI, 18.9-29.5) whose BM samples remained positive by FCM. Twenty and 10 patients relapsed in MRD positive and negative group, respectively. There was a statistically significant difference in between the 2 groups (P=0.002) (Fig. 3). There was a statistically significant

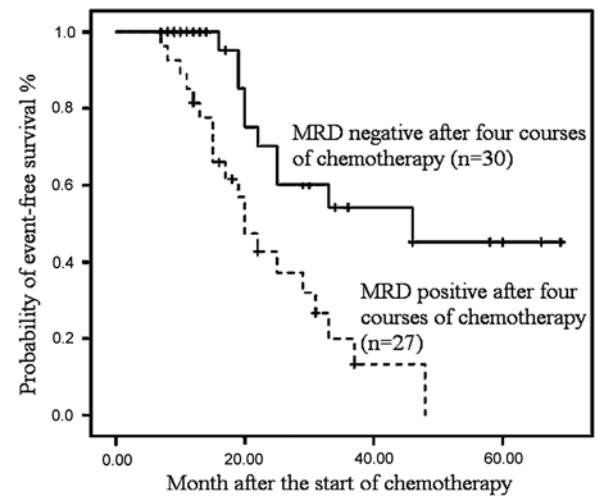

FIGURE 3. Event-free survival curves constructed with the Kaplan-Meier method. Dotted line indicates the patients whose bone marrow samples remained positive minimal residual disease (MRD) by flow cytometry after 4 courses of chemotherapy (n = 27, mean follow-up of 24.2 mo, ranging from 8 to 48, 95% confidence intervals 18.9-29.5). Solid line indicates the patients whose bone marrow samples became negative at that time (n = 30, mean follow-up of 45.5 mo, ranging from 7 to 69, 95% confidence intervals, 35.3-55.8). The difference in event-free survival between these 2 groups is significant using the log-rank test (P = 0.002).

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TABLE 2. Patient Characteristics and Disease-related Features

Patient Category	MRD Positive After 4 Courses of Chemotherapy (n = 27) No. Patients (%)	MRD Negative After 4 Courses of Chemotherapy (n = 30) No. Patients (%)	*P*
Sex			0.706
Male	17 (62.9)	18 (60)	
Female	10 (37.1)	12 (40)	
Disease status (CR/VGPR)	23 (85.2)	28 (93.3)	0.001
PBSCT	15 (55.5)	10 (33.3)	0.4
BM involve by morphology at diagnosis	26 (96.3)	28 (93.3)	0.675
BM involve by morphology after 4 courses of chemotherapy	5 (18.5)	0 (0)	0.474
Age at diagnosis (mo)			0.849
Mean	35.85	47.67	
Median	35.33	43.33	
Range	5 ~ 96	7 ~ 138	
No. cycles of chemotherapy			0.810
Mean	9.74	9.03	
Median	9.78	8.92	
Range	5 ~ 17	6 ~ 12	

BM indicates bone marrow; CR, complete response; MRD, minimal residual disease; PBSCT, peripheral blood stem cell transplantation; VGPR, very good partial response.

difference on disease remission status ($P = 0.001$). There were no difference in these 2 groups on sex, received PBSCT or not, age at diagnosis, and the number of total courses of chemotherapy (Table 2). Three of 57 BM samples are morphologically negative but positive for $CD45^-/CD81^+/CD56^+$ by FCM at diagnosis, and there is, also, no significant difference between these 2 groups (Table 2).

DISCUSSION

FCM has been used in the clinical practice of hematology and oncology for many years and has some advantages compared with immunocytochemistry or RT-PCR.[11] One specific advantage of FCM over PCR based assays is that it allows direct quantification of MRD rather than extrapolating it from amounts of PCR products.[12] This makes quantification easier and more accurate. FCM assays also have limitations. In contrast to immunocytochemistry or RT-PCR, it is virtually impossible to detect 1 NB cell in 10^6 or more mononuclear cells (0.0001%).[13] Tsang et al[14] compared the triple-color $CD45^-/CD81^+/CD56^+$ FCM assay with tyrosine hydroxylase RT-PCR and found a significant correlation between both the techniques. When FCM results were compared with immunocytochemical data, a strong correlation between both the assays was found.

Despite all the technical progress, which has been made to detect small number of circulating tumor cells, the clinical significance of these observations remains unclear. Seeger et al[15] carried out the so far largest study on 466 patients with metastatic disease by immunocytology. Focusing on 3-year EFS, they concluded that a cutoff of 100 tumor cells per 10^5 BM cells before therapy, after 12 weeks, at BM harvest and at the end of induction had a statistically significant predictive value. In a recent study, Modritz et al[16] applied the combined immunocytologic detection method to address the prognostic significance of BM clearance in stage 4 patients at a detection level of 1 in 10^6 mononuclear BM cells. With regard to 5-year survival, a statistically significant difference was found between patients obtaining BM clearance within 4 cycles of chemotherapy and those who did not.

In conclusion, the use of MRD detection by FCM for BM sample may not only allow predicting and stratifying patients for prognosis and treatment but also provide means for assessing the effect of novel therapies against MRD without having to wait for the larger tumor burden, which could be detected by routine clinical methods.

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